

# Seasonal Fluctuations of Sex Steroid Hormones in Indian Major Carp *Catla Catla* in Khouzestan, Iran

Homayoun Hosseinzadeh Sehafii<sup>1</sup>, Mozghan Khodadadi<sup>2</sup> and Shima Ayati Behbahani<sup>3\*</sup>

<sup>1</sup>Country Fisheries Research Organization, Tehran, Iran

<sup>2</sup>Aquaculture Department, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran

<sup>3</sup>Aquaculture Department, Khouzestan Science and Research Branch, Islamic Azad University, Ahvaz, Iran

## Abstract

*Catla (Catla catla)* is one of the most important species of cyprinids in terms of economic and nutritional value that was considered as aquaculture candidate in Khozaestan province (Iran). In this study, 40 female and male *Catla* broodstocks (imported from India), were studied during year (summer 2011 to spring 2012). Seasonally, blood sampling was collected with heparinated syringe followed by catching and anesthesia. Levels of steroid hormones (testosterone, 17 $\beta$ -estradiol and progesterone) were measured by RIA. Results showed significant differences in all sex steroids during seasonal sampling ( $P < 0.05$ ). High plasma testosterone levels were measured in both sexes (female:  $0.13 \pm 0.03$  ng/ml and male:  $0.16 \pm 0.04$  ng/ml) in the course of the sexual maturation at the end of winter. Highest female plasma levels of 17  $\beta$ -estradiol ( $128.50 \pm 26.60$  ng/ml) was measured in fall. Progesterone also significantly increased at winter ( $1.32 \pm 0.22$  ng/ml) in female broodstocks ( $P < 0.05$ ). All broodstocks were ripe and ready to breeding in winter. Water temperature was high ( $29.88 \pm 1.42$ ) in summer which declined to ( $14.63 \pm 0.47$ ) in winter. According to the results, climatic conditions (Temperature) had a great impact on broodstocks Gonad development via involving of hormones in Khuzestan.

**Keywords:** *Catla catla*; Seasonal changes; Steroid hormone; Environment

## Introduction

Reproduction is an important factor ensuring the continuation of a species by recruitment of the next generation; and Successful reproduction in seasonally breeding vertebrates depends on temporal variations in gametogenesis, reproductive behaviors, and steroid hormone production [1].

In generally, Reproduction in teleosts, as in other vertebrates, is under hormonal regulation by the brain-pituitary-gonadal axis (BPG axis). The main factors involved in the regulation of reproductive function are pituitary gonadotropins and gonadal steroids. Hormones are regulators which affect cells metamorphism. Hormones are not beginner of reaction and many physiological actions undergo without them. Sex steroids have long been recognized as key hormones regulating sexual differentiation, physiological aspects of reproduction and the development of primary and secondary sexual characteristics [2].

A major estrogen, 17 $\beta$ -estradiol (E2), controls pivotal physiological events in female reproductive cycles in all vertebrates studied to date. The association of changes in gonadal development with plasma levels of sex steroids has proven to be a valuable tool for understanding the endocrine control of reproduction in teleosts. In female teleosts, estradiol-reported that plasma 17 $\beta$ -estradiol (E2) levels increased during the vitellogenic stage, but decreased during the maturational stage [3]; while in male teleosts, both 11-ketotestosterone (11-KT) and testosterone (T) may regulate reproductive morphology and behavior [4]. In many if not all teleosts, progesterone can induce oocyte maturation [5]. The endocrine control cannot continue without appropriate environmental cues required stimulating reproduction [6]. Many external or environmental cues can have profound effects on circulating hormone levels. For example, water temperature, photoperiod, lunar and tidal cycles, and food availability are known to influence spawning behavior, gametogenesis, and circulating steroid

levels in fishes [7,8]. Sexual maturity and gonadal development is associated with increased circulating levels of gonadotropins and plasma sex steroids [9].

The major carp *Catla (Catla catla)* (Cyprinidae, Cypriniformes) is a popular freshwater edible fish and an extensively cultured species in India. This species has been investigated to study its taxonomy, ecology, abundance, feeding and growth parameters, but to date, reproductive biology is quite neglected in many areas [10]. Recently, the exploitation of several valuable cyprinid species for commercial purposes has made their investigation particularly relevant [11]. *Catla* is a species of choice for the study of temporal organization of reproduction under natural and experimental conditions for understanding environmental and/or endocrine mechanisms that control the annual reproductive cycle [12].

Seasonal changes in the concentrations of circulating sex hormones and their importance for reproduction has been reported for several species of teleosts [13]. Different study have been accomplished on seasonal fluctuation of sex steroid hormone levels in different species like vocal plainfin midshipman [14], *Epinephelus coioides* [15], *Leuciscus cephalus* [16], *Labeo rohita* [17], *Huso huso* [18], *Esox lucius* [19].

The aim of this study was to investigate the seasonal fluctuations of plasma sex steroids in male (testosterone), and female (testosterone, 17 $\beta$ -estradiol and progesterone) *Catla (Catla catla)* in Khouzestan.

**\*Corresponding author:** Shima Ayati Behbahani, Islamic Azad University, Aquaculture Branch, Khouzestan Science and Research Branch, Islamic Azad University, Ahvaz-Iran, Tel: +989163718056; Fax: +98-21-22492794; E-mail: [ayati.shilat@yahoo.com](mailto:ayati.shilat@yahoo.com)

Received March 21, 2014; Accepted May 28, 2014; Published June 02, 2014

**Citation:** Sehafi HH, Khodadadi M, Behbahani SA (2014) Seasonal Fluctuations of Sex Steroid Hormones in Indian Major Carp *Catla Catla* in Khouzestan, Iran. J Environ Anal Toxicol 4: 227. doi: [10.4172/2161-0525.1000227](https://doi.org/10.4172/2161-0525.1000227)

**Copyright:** © 2014 Sehafi HH, et al.. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

## Materials and Methods

### Fish supply

The study was conducted between summer 2011 and spring 2012. Totally 40 *Catla* Broodstocks (5 female and 5 male) were captured from earthen ponds in each season. Fishes were imported from India at 2010 and transported to South Iran Aquaculture Research Center (31° 40' N, 48° 79' E), Ahvaz (Khouzestan Province, Iran). Fish were fed with concentrates fishes (Byza Fars Company) two a day.

### Blood sampling

Five samples of both male and female broodstocks were captured seasonally. Fishes were recaptured in each season according to tagged fishes (ABC elastomer Tag, CIST, Canada). The blood samples were taken from caudal vein via heparinized syringe. Plasma was extracted using centrifuge (10 min. at 3000×g) and stored at -20°C until hormone assay [20]. Testosterone (for male and females), 17β-estradiol and progesterone (for females) were measured with radioimmunoassay political approach (RIA) by the commercial Kit of Finland Immune Tech (I<sup>125</sup>) and gamma counter [21].

### Hormone assays

RIA is a quantitative immunoassay technique used to detect the level of protein or antigen in a sample by measuring the diameter of the ring of precipitin formed by the complex of the protein and the antiserum. To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine attached to tyrosine [22].

This radiolabeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two chemically bind to one another. Then, a sample of serum from containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radiolabeled antigen ("hot") for antibody binding sites. As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radiolabeled variant, and reducing the ratio of antibody-bound radiolabeled antigen to free radiolabeled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigen remaining in the supernatant is measured using a gamma counter. Using known standards, a binding curve can then be generated which allows the amount of antigen (IgM) in the serum to be derived [23].

### Water temperature

Pond water temperature was monthly measured by thermometer. Average water temperature was calculated for each season according to 3 depth data collection.

### Statistical analysis

Normality of data was tested by Kolmogorof-Smirnof test. All data were expressed as means ± S.E.M.

The SPSS 15 software was used for statistical analyses and Excel 11 for graphs. Differences between groups were evaluated by analysis of variance (one way ANOVA). The significant differences were determined at (P<0.05).

## Results

### Testosterone (T)

In the present study level of testosterone hormone in female in spring, summer, autumn and winter seasons were 0.1 ± 0.08 (ng/ml), 0.04 ± 0.02 (ng/ml), 0.1 ± 0.07 (ng/ml) and 0.13 ± 0.03 (ng/ml) respectively, that higher level belong to winter season. There was significant difference in level of testosterone among spring and fall with summer season (P<0.05). Moreover, there was significant difference in plasma testosterone levels between all seasons with winter season (P<0.05). Lower level of testosterone was observed in summer season (Figure 1).

According to the results, level of testosterone hormone in male in spring, summer, autumn and winter seasons were 0.05 ± 0.02 (ng/ml), 0.04 ± 0.02 (ng/ml), 0.04 ± 0.05 (ng/ml) and 0.16 ± 0.04 (ng/ml) that higher level belong to winter season and there was significant difference in level of testosterone among winter season with spring, summer and fall (P<0.05). Also, lower this levels of testosterone in male, was observed in summer season (Figure 1).

### Estradiol (E2)

17β-estradiol hormone analysis during four seasons showed Level of 17β-estradiol hormone in spring, summer; autumn and winter seasons were 85.50 ± 16.68 (ng/ml), 101.50 ± 55.23 (ng/ml), 128.50 ± 26.60 (ng/ml) and 88.75 ± 9.50 (ng/ml). There was significant difference in level of 17β-estradiol between spring and winter with summer

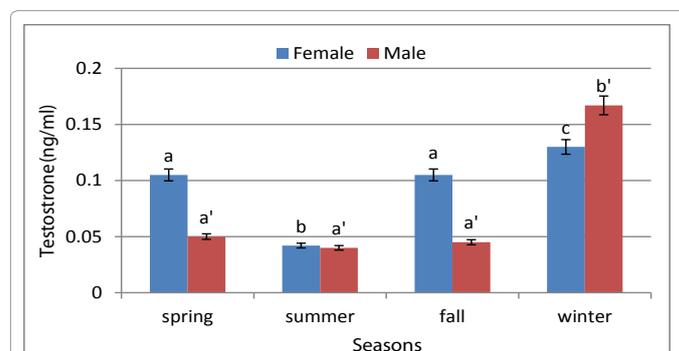


Figure 1: Changes testosterone (T) levels in female and male *catla catla* fish in seasons (Mean ± S.E.M.)

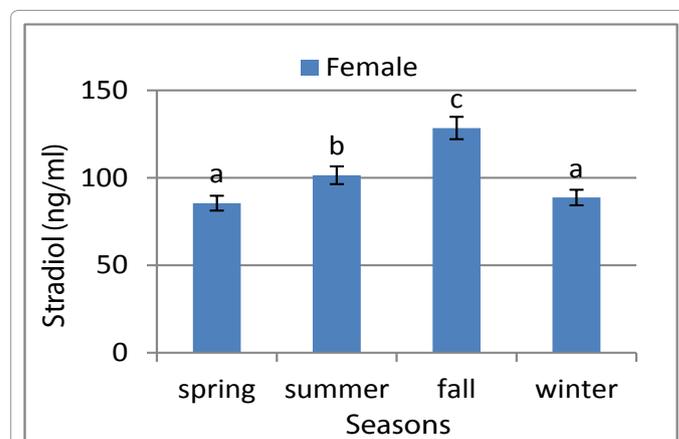


Figure 2: Changes 17β-Estradiol (E2) levels in female *catla catla* fish in seasons (Mean ± S.E.M.)

season ( $P < 0.05$ ). Also, There was significant difference between All seasons with fall ( $P < 0.05$ ). Highest of  $17\beta$ -estradiol level was observed in fall season. Amount of  $17\beta$ -estradiol was measured only in female (Figure 2).

### Progesterone (P)

In the case of progesterone hormone, higher levels were seen winter season and there was significant difference between winter season with other seasons ( $P < 0.05$ ). Levels of progesterone hormone in spring, summer, autumn and winter seasons were  $0.38 \pm 0.18$  (ng/ml),  $0.51 \pm 0.4$  (ng/ml),  $0.46 \pm 0.13$  (ng/ml) and  $1.32 \pm 0.22$  (ng/ml). This hormone was measured only in female (Figure 3).

### Temperature

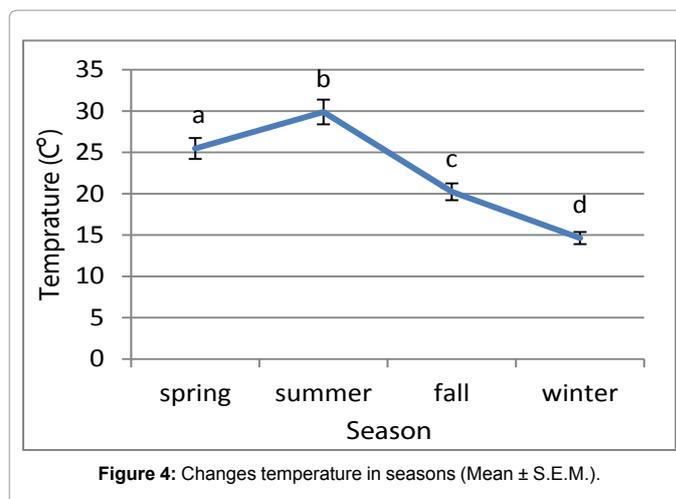
Average water temperature is measured at different seasons, were obtained as follows (Figure 4). Spring ( $25.47 \pm 1.07$ ), summer ( $29.88 \pm 1.42$ ), fall ( $20.23 \pm 0.69$ ), winter ( $14.63 \pm 0.47$ ). Each season, there was a significant difference with the other seasons ( $P < 0.05$ ).

### Discussion

Seasonal changes in the concentrations of circulating sex hormones and their importance for reproduction has been reported for several species of teleosts. Studies have shown that annual fluctuations of hormones related to reproductive, feeding and growth cycles in fishes [15]. Annual rhythm of hormones closely related to factors such as temperature, environment, species of fish, length of day and gonadal sex steroids [2].

Reproduction in fish is under hormonal regulation by the hypothalamus–pituitary–gonadal axis. The brain is stimulated by environmental cues like flooding, water temperature, feeding, rainfall and photoperiod to release gonadotropin releasing hormone [24]. In the pituitary, Gonadotropin Releasing Hormone (GnRH) stimulates the synthesis of Gonadotropin (GtH). GtH-I (or FSH), involved in the initial stages of gametogenesis (vitellogenesis and spermatogenesis), and GtH-II (or LH), which sets FOM, spermiogenesis and spermiation [25]. GtH-I and GtH-II have distinct temporal expression and release profile in teleosts. GtH-I is released over the entire vitellogenesis, whereas GtH-II remains low during vitellogenesis and exhibit a sharp peak before ovulation [26].

The present study was undertaken to investigate changes of serum sex steroids (E2, T and P) and in both sexes *Catla catla* during the



seasonally reproductive cycle. Seasonal changes in circulating plasma levels of sex steroid hormones during the reproductive cycle are described for a variety of teleost species [27,28].

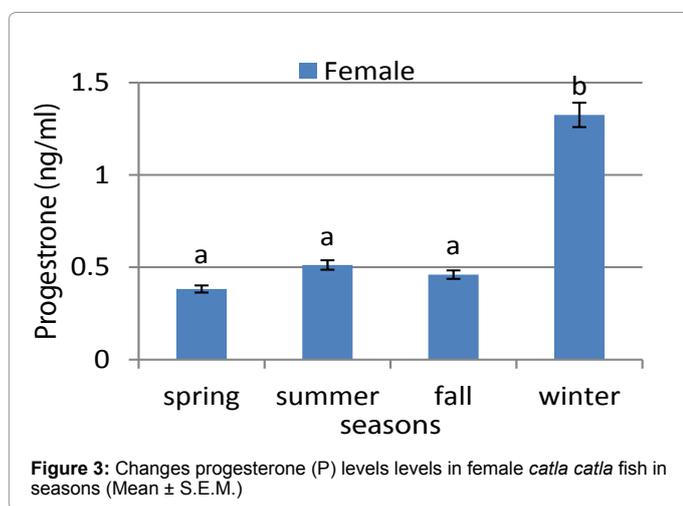
Estradiol is known to be secreted by the cells of the ovarian follicles that promote the development and maintenance of the female sexual characteristics. In humans this hormone (together with other hormones) is responsible for controlling the female sexual cycle. In a variety of species, the level of serum E2 begins to increase in accordance with the appearance of active vitellogenic oocytes, and reaches the highest levels in the tertiary yolk stage oocyte in the ovary, and sharply declines in fish with postvitellogenic and atretic ovaries [29].

According to the results, Plasma E2 levels peak towards the end of vitellogenesis at fall ( $128.50 \pm 26.60$  ng/ml) at temperature ( $20.23 \pm 0.69$ ), and they decline rapidly in the maturation stage ( $88.5 \pm 9.50$  ng/ml) in winter. Similarly, a peak in  $17\beta$ -estradiol plasma concentrations associated with the synthesis of vitellogenic proteins (vitellogenin) has been described in many other teleosts [2]. This result coincided with other studies such as study on Common carp [1], *Acipenser Persicus* [30], *Rutilus firrsi kutum* [31], *Labeo rohita* [32]. In these studies, levels of estradiol had difference during year and its peak was before spawning.

Statistical results showed testosterone at both sex in winter. Along with the increase in water temperature in spring ( $25.47 \pm 1.07$ ), was more than other seasons (the stage of sexual maturation); which coincides with after vitellogenesis. Testosterone (T) has been identified in plasma of female teleosts and may be utilized as the major aromatizable androgen [17].

In male teleosts, testosterone is typically elevated during spermatogenesis, and then falls at the onset of spermiation [33]. It appears that a time lapse exists between testosterone production and the male breeding cycle, which is associated with female gonadal maturity. Present result coincided with other researches like study on *Rutilus firrsi kutum* [3], *Capoeta capoeta umbla* [34], *Acanthopagrus butcheri* [35].

Fluctuations in  $17\beta$ -estradiol paralle led those of androgens, suggesting a close relationship between these steroids. Although the primary role of E2 is to aid in gonadal development, T is involved in other functions, such as positive and negative feedback control of the hypothalamus–pituitary–gonadal axis and migratory behavior in sturgeons [36]. *In vitro* experiments have shown that androgens are a



substrate for 17 $\beta$ -Estradiol production in both male and female teleosts [37].

Also the highest level of progesterone level (1.32  $\pm$  0.22 ng/ml) was observed in winter. In female fish, levels of maturation-inducing steroids (usually progesterone) are elevated during final oocyte maturation and ovulation [35]. Progesterone increases in a short period, can indicate a limited role of this hormone on ovarian function and also indirectly involved in the final maturation ovary by hydroxyprogesterone [32]. Levels of progesterone increased with progression of gonadic stage in *Huso huso* [18].

It is known that the role of sex steroids in controlling the maturation cycle in teleosts especially during spawning times is altered by environmental or hormonal manipulation, and this has both theoretical and practical relevance [38]. Based on the results of this study seem, sex steroid hormones during spawning are changed, the change of seasons and the environment is very effective.

However, due to the great influence of environmental conditions can be said to Khouzestan with suitable climatic conditions such as temperature, the influence of hormonal changes and increase their levels, is considered a good place to culture this fish.

## References

1. Taghzadeh V, Imanpoor MR, Mehdinejad N (2013) Study the seasonal steroid hormones of common carp in Caspian Sea, Iran. Springerplus 2: 193.
2. Pavlidis M, Greenwood L, Mourot B, Kokkari C, Le Menn F, et al. (2000) Seasonal variations and maturity stages in relation to differences in serum levels of gonadal steroids, vitellogenin, and thyroid hormones in the common dentex (*Dentex dentex*). Gen Comp Endocrinol 118: 14-25.
3. Shafiei sabet S, Imanpour MR, Aminain Fatideh B, Gorgin S (2010) Study on levels of 17 $\beta$ estradiol and testosterone in Female Kutum *Rutilus Frisii kutum* (kamenshii,1901) of southern Caspian sea. World journal of Zoology 5: 217-224.
4. Fostier A, Le Gac F, Loir M (1987) Steroids in male reproduction. In: Idler, D.R., Crim, L.W., Walsh, J.M. (Eds.), Proceedings of the Third International Symposium on Reproductive Physiology of Fish. Memorial University, St John's, Canada 239-245.
5. Kobayashi D, Tanaka M, Fukada S, Nagahama YS (1996) Steroidogenesis in the ovarian follicles of the medaka (*Oryzias latipes*) during vitellogenesis and oocyte maturation. Zoological Science 13: 921-927.
6. Fahmy AF (2006) Induced spawning in one species of family Bagruidae. Ph.D. Thesis. Faculty of Science. Zagazig University.
7. Takemura A, Rahman Md, Nakamura S, Park YJ, Takano K (2004) Lunar cycles and reproductive activity in reef fishes with particular attention to rabbitfishes. Fish Fisheries 5: 317-328.
8. Wang Q, Hong W, Chen S, Zhang Q (2008) Variation with semilunar periodicity of plasma steroid hormone production in the mudskipper *Boleophthalmus pectinirostris*. General and Comparative Endocrinology 155: 821-826.
9. Huggard D, Khakoo Z, Kassam G, Mahmoud SS, Habibi HR (1996) Effect of testosterone on maturational gonadotropin subunit messenger ribonucleic acid levels in the goldfish pituitary. Biol Reprod 54: 1184-1191.
10. Ayyappan S, Jena JK (2003) Grow-out production of carps in India. Journal of Applied Aquaculture 13: 251-282.
11. Berry C (2003) Fishy business. QJM 96: 83-84.
12. Maitra SK, Chattoraj A (2012) Role of photoperiod and melatonin in the regulation of ovarian functions in Indian carp *Catla catla*: basic information for future application. Fish Physiology and Biochemistry 33: 367-382.
13. Barannikova IA, Dyubin VP, Bayunova LV, Semenkova TB (2002) Steroids in the control of reproductive function in fish. Neurosci Behav Physiol 32: 141-148.
14. Sisneros JA, Forlano PM, Knapp R, Bass AH (2004) Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman. Gen Comp Endocrinol 136: 101-116.
15. Abbasi F, Oryan S, Matin Far A (2008) The changes in sex hormones during ovarian development stages of *Epinephelus coioides* in Persian Gulf. Iranian Journal of Research and Development in Livestock and Aquaculture 79: 72-80.
16. Guerriero G, Ferro R, Ciarcia G (2005) Correlations between Plasma Levels of Sex Steroids and Spermatogenesis during the Sexual Cycle of the Chub, *Leuciscus cephalus* L. (Pisces: Cyprinidae). Zoological Studies 44: 228-233.
17. Sen U, Mukherjee D, Bhattacharyya SP, Mukherjee D (2002) Seasonal changes in plasma steroid levels in Indian major carp *Labeo rohita*: influence of homologous pituitary extract on steroid production and development of oocyte maturational competence. Gen Comp Endocrinol 128: 123-134.
18. Alizadeh M, Hedayati AA, Bahmani M, Jamalzadeh HR (2010) Seasonal fluctuations of steroid hormones in beluga sturgeon *Huso huso* cultured in brackish water. International Aquatic Research 2: 121-126.
19. Khodadoust A, Imanpoor MR, Zadeh VT, Khara H, Rahbar M, et al. (2013) Study on Levels of Sex Steroid Hormones of Pike (*Esox lucius* Linnaeus, 1758) in Anzali Wetland. Global Veterinaria 10: 354-359.
20. Pottinger TG, Carrick TR (1991) A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress response in female rainbow trout. Aquaculture 175: 351-363.
21. Avella M, Young G, Prunet P, Schreck CB (1990) Plasma prolactin and cortisol concentrations during salinity challenges of coho salmon (*Oncorhynchus kisutch*) at smolt and post-smolt stages. Aquaculture 91: 359-372.
22. Werner SC, Acebedo G, Radichevich I (1974) Rapid radioimmunoassay for both T4 and T3 in the same sample of human serum. J Clin Endocrinol Metab 38: 493-495.
23. Yalow RS, Berson SA (1960) Immunoassay of endogenous plasma insulin in man. J Clin Invest 39: 1157-1175.
24. Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O (2010) Neuroendocrinology of reproduction in teleost fish. Gen Comp Endocrinol 165: 438-455.
25. Mylonas CC, Magnus Y, Gissis A, Klebanov Y, Zohar Y (1996) Application of controlled-release, GnRH $\alpha$ -delivery systems in commercial production of white bass X striped bass hybrids (sunshine bass), using captive broodstocks. Aquaculture 140: 265-280.
26. Prat F, Sumpster JP, Tyler CR (1996) Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biology of Reproduction 54: 1375-1382.
27. Kousha A, Askarian F, Yousefian M, Ghate HV, Ghole VS (2009) Annual Fluctuation of Sex Steroid Hormones in Pre-spawning Female Kutum (*Rutilus frisii kutum*). World Journal of Fish and Marine Sciences 1: 65-73.
28. Bahmani, M, Jourdehi AY, Kazemi R, Pourdehghani M, Hallajian A, et al. (2009) Seasonal fluctuations of sex steroids (Testosterone, 17  $\alpha$ -hydroxy progesterone and 17  $\beta$ -estradiol) with sexual development in farmed Stellate sturgeon, *Acipenser stellatus*. Iranian Scientific Fisheries 17: 7-16.
29. Matsuyama M, Adachi S, Nagaham Y, Kitajima C, Matsuura M (1991) Annual reproductive cycle of the captive female Japanese sardine *Sardinops melanostictus*: relationship to ovarian development and serum levels of gonadal steroid hormones. Marine Biology 108: 21-29.
30. Hosseinzade M, Imanpoor MR, Aghilinejad SM (2012) Changes in Serum Steroid Hormones by Ovarian Development of Persian Sturgeon (*Acipenser Persicus*) during Final Maturation Induced by Hormonal Treatment. Scientific Reports 1: 532.
31. Heidari B, Roozati SAA, Yavari L (2010) Changes in plasma levels of steroid hormones during oocyte development of caspian kutum (*Rutilus frisii kutum*). Anim Reprod 7: 373-381.
32. Suresh DV1, Baile VV, Prasada Rao PD (2008) Annual reproductive phase-related profile of sex steroids and their carrier, SHBG, in the Indian major carp, *Labeo rohita*. Gen Comp Endocrinol 159: 143-149.
33. Fostier A, Jalabert B, Billard R, Breton B, Zohar Y (1983) The gonadal steroids. In: W.S. Hoar. D.J. Randall and E.M. Donaldson (Editors). Fish Physiology 11: 277-332.
34. Erdogan O, Haliloglu HI, Ciltas A (2002) Annual Cycle of serum gonadal steroids and serum lipids in capoeta capoeta umbla (Gulden staedt) 1772 (Pisces: Cyprinidae). Turkish Journal of Veterinary and Animal Sciences 26: 1093-1096.

- 
35. Hobby AC, Geraghty DP, Pankhurst NW (2000) Differences in Binding Characteristics of Sex Steroid Binding Protein in Reproductive and Nonreproductive Female Rainbow Trout (*Oncorhynchus mykiss*), Black Bream (*Acanthopagrus butcheri*) and Greenback Flounder (*Rhombosolea tapirina*). General and Comparative Endocrinology 120: 249-259.
36. Talbott M, Van Eenennaam P, Linares-Casenave J, Doroshov I, Guy S, et al. (2011) Investigating the use of plasma testosterone and estradiol-17 $\beta$  to detect ovarian follicular atresia in farmed white sturgeon, *Acipenser transmontanus*. Aquaculture 315: 283-289.
37. Barannikova IA1, Diubin VP, Baiunova LV, Semenkova TB 2000 Steroids in the reproductive function regulation in fish. Ross Fiziol Zh Im I M Sechenova 86: 968-978.
38. Flammarión P, Brion F, Babut M, Garric J, Migeon B, et al. (2000) Induction of fish vitellogenin in testicular structure: preliminary results of estrogenic effects in chub. Ecotoxicology 9: 127-135.